

Involvement of the Glycoprotein Ib-V-IX Complex in Nickel-Induced Platelet Activation

Silvia Riondino, Fabio Maria Pulcinelli, Pasquale Pignatelli, and Pier Paolo Gazzaniga

Department of Experimental Medicine and Pathology, University of Rome La Sapienza, Rome, Italy

We studied the effect of nickel ions on platelet function because hypernickelemia has been found in patients with acute myocardial infarction. We previously demonstrated that nickel can activate an intracellular pathway leading to cytoskeleton reorganization consequent to tyrosine phosphorylation of p60^{src} in human platelets independently of integrin alpha-IIb-beta₃ (αIIbβ₃). Moreover, in von Willebrand factor-stimulated platelets, the tyrosine phosphorylation of pp60^{c-src} is closely associated with the activation of phosphatidylinositol 3-kinase (PIK), and two adhesion receptors, glycoprotein (Gp)Ib and GpIIb/IIIa (αIIbβ₃), are involved. In our study, 1 and 5 mM nickel in the presence of fibrinogen induced platelet aggregation (independently of protein kinase C activation) and secretion. The pretreatment with a PIK inhibitor, wortmannin, strongly decreased nickel-induced platelet aggregation. Platelet treatment with mocoarhagin, a cobra venom metalloproteinase that cleaves GpIb, significantly reduced aggregation induced by 5 mM without affecting the response to other agonists such as adenosine diphosphate (ADP). Moreover, nickel caused PIK translocation to the cytoskeleton. Taken together, these observations suggest a partial involvement of both integrins αIIbβ₃ and GpIb-V-IX complex in Ni²⁺-induced platelet activation. **Key words:** adhesion receptors, integrins, nickel, platelet activation. *Environ Health Perspect* 109:225–228 (2001). [Online 26 February 2001] <http://ehpnet1.niehs.nih.gov/docs/2001/109p225-228riondino/abstract.html>

The mechanism of action of nickel compounds in the pathogenesis of several diseases linked to occupational exposure has been analyzed, but the toxicity, uptake, and mutagenicity of nickel are not fully understood. Nickel compounds exhibit differential activities at the level of cell surface; consequently, physicochemical surface interactions might contribute to cell injury in nickel-induced cytotoxicity (1). Moreover, nickel can induce in several human and rodent cell lines the expression of the receptor system Cap43, which is involved in a Ca²⁺-dependent process of signal transduction (2–4). Nickel induces CD69 expression in lymphocyte subpopulations of allergic patients with contact dermatitis (5). CD69 is constitutively expressed on human platelet surface where it can be involved in signal transduction (6). Hypernickelemia has been found in patients with acute myocardial infarction and has been thus related to the pathogenesis of myocardial ischemic injuries (7), in which platelet interactions with the exposed collagen fibers play a major role.

In previous studies we showed that NiCl₂ can enhance platelet aggregation induced by collagen in the presence of fibrinogen via a rapid cytoskeletal reorganization consequent to tyrosine phosphorylation of pp60^{src}, a signaling molecule that has been detected in a submembraneous location (8). However, nickel-induced platelet activation, expressed as p60^{src} phosphorylation, occurs even in the absence of fibrinogen, whereas aggregation requires fibrinogen binding to its receptor, integrin αIIbβ₃ (8).

The pp60^{src} protein is not the only protein involved in regulating the formation of platelet cytoskeletal signaling complexes. Calpain, a thiol protease, is responsible for the cleavage of several adhesion structural proteins (talin and integrin αIIbβ₃) (9,10) and signaling enzymes [focal adhesion kinase (FAK); phosphatidylinositol 3-kinase (PIK)] (11,12) in spreading and aggregating platelets.

Von Willebrand factor (vWf)-induced platelet stimulation requires the coactivation of two different glycoproteins, the GpIb-V-IX complex and GpIIb-IIIa, or integrin αIIbβ₃, the former being responsible for the initial contact and the latter leading to spreading and irreversible adhesion (13). However, vWf binding to GpIb-V-IX complex induces the activation and cytoskeletal association of pp60^{src} and PIK (14); this behavior resembles that observed in NiCl₂-treated platelets.

Our aim was thus to verify whether the GpIb-V-IX complex has a role in NiCl₂-induced platelet activation and to investigate the molecules involved in the signal transduction cascade that follows NiCl₂ binding to platelet membrane.

Materials and Methods

Materials. Luciferin and luciferase were obtained from Chrono-Log (Havertown, PA, USA). HEPES, fibrinogen, Arg-Gly-Asp-Ser (RGDS) peptide, bovine serum albumin (V-BSA), glucose, wortmannin, ADP, and U46619 were obtained from Sigma (St. Louis, MO, USA). Calpeptin and Ro 31-8220 were obtained from Calbiochem-Novabiochem (La Jolla, CA,

USA). Mocoarhagin, lyophilized venom from *Naja mossambica mossambica*, was obtained from Latoxan (Rosans, France). And anti-PIK, p85 (rabbit polyclonal IgG), was obtained from Upstate Biotechnology (Lake Placid, NY, USA).

Platelet isolation. We collected human platelets from drug-free, healthy donors in acid/citrate/dextrose (ACD)-containing tubes. Platelet-rich plasma (PRP) was obtained after centrifugation at 180 × g for 15 min and concentrated at 800 × g for 20 min. The platelet pellet was resuspended in one-third volume of autologous platelet-poor plasma (PPP) and incubated with 1 mM aspirin for 15 min at 37°C. The platelets were then washed twice in Tyrode's buffer (137 mM NaCl, 2.68 mM KCl, 0.42 mM NaH₂PO₄, 1.7 mM MgCl₂) containing 10 mM HEPES (pH 6.5) and resuspended in Tyrode's buffer containing 0.2% BSA, 0.1% glucose, and 10 mM HEPES (pH 7.35). The final platelet suspension was adjusted to 2.5 × 10⁸ cells/mL.

Platelet aggregation. *In vitro* platelet aggregation was performed in a PACKS-4 aggregometer (Helena Laboratories, Beaumont, TX, USA) using siliconized glass cuvettes at 37°C under continuous stirring. NiCl₂ (1 mM and 5 mM), ADP (5 μM), and the thromboxane A₂ analog U46619 (1 μM) were used as platelet agonists. Fibrinogen (1 mg/mL) was added before the agonists.

ATP release. Platelet activation was stopped after 2 min by adding formaldehyde/EDTA according to Costa and Murphy (15). After centrifugation at 10,000 × g for 30 sec, we measured the ATP concentration in the supernatant in an LKB 1251 luminometer (LKB, Turku, Finland) after adding luciferin (40 mg/mL) and luciferase (880 U/mL). The results were expressed as the percentage of ATP released relative to the total ATP present in cells lysed by means of digitonin (50 μM) (16).

Address correspondence to P.P. Gazzaniga, Department of Experimental Medicine and Pathology, University of Rome La Sapienza, Viale Regina Elena 324, 00161, Rome, Italy. Telephone: +39-064452955. Fax: +39-064454820. E-mail: pierpaolo.gazzaniga@uniroma1.it

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Mocarhagin purification. We purified mocarhagin from the crude venom of the snake *Naja mossambica mossambica* for its heparin binding properties according to De Luca et al. (17). Briefly, crude lyophilized venom (0.5 g) was dissolved in water (10 mL) and loaded onto a heparin-sepharose CL-6B column (1.5 × 40 cm; Pharmacia, Uppsala, Sweden) at 25 mL/hr. After washing with the column buffer containing 0.01 M Tris, 0.15 M sodium chloride, pH 7.4, bound protein was eluted with a linear 250-mL, 0.15–1.0 M sodium chloride gradient in 0.01 M Tris, pH 7.4. Fractions containing mocarhagin were identified by HPLC gel-filtration using a G2000SWXL column (15 mm, 30 cm × 7.8 mm; Supelco Inc., Bellefonte, PA, USA), pooled, ultraconcentrated, and then loaded at 25 mL/hr onto a sepharose CL-6B column (1.5 × 70 cm; Pharmacia). Peak eluted fractions were dialyzed against the column-washing buffer. Mocarhagin (10 µg/mL) was added to platelets 15 min (at 37°C) before agonist stimulation.

Cytoskeleton studies. Aliquots of 500 µL of aspirinated peptide RGDS-treated platelet suspensions (2.5×10^8 cells/mL) were stimulated with 5 mM nickel. After 1, 3, and 5 min the reactions were stopped by the addition of an equal volume of ice-cold Triton extraction buffer [2% Triton X-100, 10 mM EGTA, 0.1 mM Tris, 1 mg/mL leupeptin, 20 mM pepstatin A, 2 mM phenylmethylsulphonyl fluoride (PMSF)]. Lysates were then centrifuged at $1,500 \times g$ for 10 min to remove intact platelets. Triton-insoluble proteins were isolated by centrifugation at $15,600 \times g$ for 15 min at 4°C. Cytoskeletal proteins were separated by 10% SDS-polyacrylamide gel electrophoresis, under denaturing conditions, and then transferred to Immobilon-P (Millipore, Bedford, MA, USA) membranes. The nonspecific bindings were saturated with 3% BSA. Proteins were identified with

anti-p-85 (subunit of phosphatidylinositol 3-kinase) polyclonal antibody, followed by horseradish peroxidase-conjugated secondary antibody and visualized with ECL chemiluminescence reaction reagent (Amersham, Buckinghamshire, England) and Kodak X-ray film (X-OMAT AR; Sigma).

Results

In the presence of fibrinogen (1 mg/mL), NiCl₂ induced platelet aggregation (1 mM: $46.7 \pm 9.6\%$; 5 mM: $78 \pm 8.7\%$) (Figure 1) and released ATP from the internal stores ($25.4 \pm 4.5\%$ of the total ATP present in lysed cells vs. $3.7 \pm 1.2\%$ in the absence of fibrinogen). Control, nonstimulated platelets produced an ATP release of $2.5 \pm 0.6\%$ (Figure 2).

The treatment of washed platelets with a protein kinase C inhibitor, Ro 31-8220 (10 µM), did not modify the aggregometrical response to 1 mM NiCl₂ (data not shown) and only slightly reduced 5 mM-induced response (56.2 ± 2.5 vs. 78 ± 8.7 maximum % of aggregation) (Figure 3).

When washed platelets were treated with a calpain selective inhibitor, calpeptin (50 µg/mL), platelet aggregation in response to the thromboxane synthetic agonist U46619 (1 µM) was completely abolished (Figure 4, trace a), whereas platelet response to NiCl₂ was unmodified (Figure 4, trace b).

Figure 5 shows that when washed platelets were treated with mocarhagin (10 µg/mL), a cobra venom metalloprotease that cleaves GpIbα, the platelet response to the specific agonist of GpIbα (Ristocetin) was

completely inhibited (data not shown), whereas platelet aggregation induced by 5 mM NiCl₂ in the presence of fibrinogen was significantly reduced (37.8 ± 18.6 vs. 78 ± 8.7 maximum % of aggregation), and the response to ADP, whose action is not exerted through GpIb-V-IX, was unaffected (70.8 ± 12.3 vs. 71.4 ± 10.6 maximum % of aggregation; Figure 5).

When washed platelets were treated with a PIK inhibitor, wortmannin (10 µM), platelet aggregation induced by 1 mM NiCl₂ was completely abolished, and the aggregation induced by 5 mM NiCl₂ was strongly decreased (36.6 ± 15.9 maximum % of aggregation; Figure 6).

Figure 7 shows the immunoblot analysis of the low-speed Triton insoluble cytoskeletal fraction of nickel-stimulated platelets using a polyclonal antibody directed against the p85 subunit of PIK, closely correlated with PtdIns 3-kinase enzymatic activity in platelets (18). The figure shows that a faint band, corresponding to p-85, already detectable after 3 min NiCl₂ stimulation, became marked after 5 min stimulation of aspirinated, RGDS-treated platelets.

Discussion

In the present study we provided evidence that nickel can cause platelet aggregation and ATP release from the internal stores. NiCl₂ exerts these actions without entering the cell; in fact, in unpublished observations, we found that the addition of NiCl₂ to Fura-2-treated platelets did not quench Fura-2 fluorescence, but an evident reduction was obtained after cellular lysis. This result was obtained comparing the reduction of Fura-2 fluorescence induced by NiCl₂ to the capability of Mn²⁺ to totally quench the Fura-2 fluorescence after the addition of digitonin (50 µM), according to the method described by Sage (19).

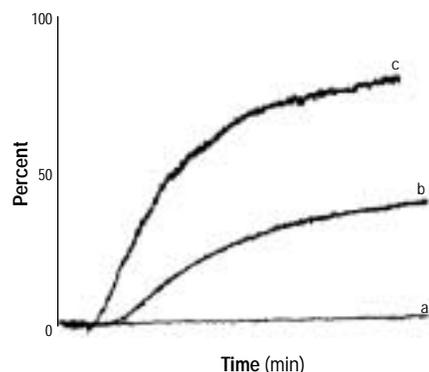


Figure 1. Platelet aggregation patterns in response to 5 mM NiCl₂ in (a) the absence of fibrinogen and in the presence of fibrinogen (1 mg/mL) with (b) 1 mM NiCl₂ and (c) 5 mM NiCl₂. The figure is representative of three experiments performed.

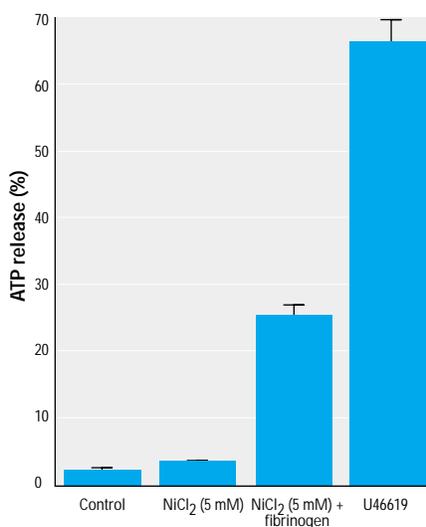


Figure 2. ATP release induced by 5 mM NiCl₂ alone, 5 mM NiCl₂ in the presence of fibrinogen (1 mg/mL), and 1 µM U46619 compared to control, unstimulated samples. The results are expressed as the mean percentage ± SE of four experiments performed of ATP released relative to the total ATP present in lysed cells. Data were analyzed by Student's *t*-test for paired data.

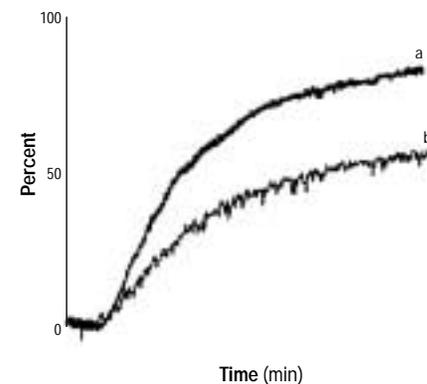


Figure 3. Platelet aggregation patterns in response to 5 mM NiCl₂ in the presence of fibrinogen (1 mg/mL) in control, washed platelets (a) and (b) after Ro 31-8220 (10 µM for 3 min at 37°C) treatment. The figure is representative of four experiments performed.

Moreover, although fibrinogen is essential for NiCl₂-induced platelet aggregation, it is not required for NiCl₂-induced platelet activation, expressed as p60^{src} phosphorylation, as demonstrated by the fact that such response is not inhibited by the incubation with the tetrapeptide RGDS (120 µg/mL), which prevents fibrinogen binding to its receptor, the integrin αIIbβ3 (8).

Many authors suggested that fibrinogen receptor exposure involves protein kinase C (20). However, NiCl₂-induced platelet aggregation does not seem to depend on protein kinase C activation, as demonstrated by the observation that the treatment with a protein kinase C inhibitor, Ro 31-8220, only slightly modified the aggregometrical response to NiCl₂.

In a previous study (8), we demonstrated that NiCl₂ induces a rapid cytoskeletal reorganization consequent to tyrosine phosphorylation of the signaling molecule p60^{src}. Among the key proteins regulating the formation of

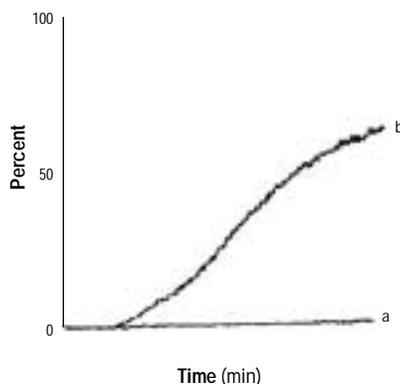


Figure 4. Platelet aggregation patterns of calpeptin-treated (50 µg/mL for 30 min at 37°C) platelets in the presence of fibrinogen (1 mg/mL) in response to (a) 1 µM U46619 and (b) 5 mM NiCl₂. The figure is representative of three experiments performed.

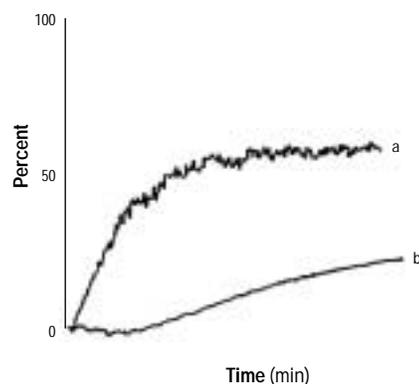


Figure 5. Platelet aggregation patterns of mocarhagin-treated (10 µg/mL for 15 min at 37°C) platelets in the presence of fibrinogen (1 mg/mL) in response to (a) 5 mM ADP and (b) 5 mM NiCl₂. The figure is representative of four experiments performed.

platelet cytoskeletal signaling process, calpain is not involved in NiCl₂-induced triggering of signal transduction. In fact, the treatment with the calpain inhibitor calpeptin did not reduce NiCl₂-induced response.

Because it has been demonstrated that pp60^{src} translocation to the cytoskeleton is triggered by vWf binding to GpIb-V-IX complex, independently of ligand binding to αIIbβ3 (14), and that neither vWf nor αIIbβ3 requires calpain activation, it was conceivable that a direct involvement of GpIb-V-IX complex in Ni²⁺ evoked platelet activation. To verify this hypothesis, we performed a platelet treatment with the cobra venom metalloprotease mocarhagin, which cleaves GpIbA. The observation of a significant reduction of platelet aggregation in response to NiCl₂ led us to hypothesize that NiCl₂ exerts its action mainly through GpIb-V-IX. Moreover, this seems confirmed by the fact that the cytoskeletal translocation of activated pp60^{src}, similar to what was observed after vWf stimulation (14), was demonstrated in NiCl₂-treated platelets (8). Because the association of p60^{src} and PIK to cytoskeleton occurs once platelets aggregate (14), we tested whether the activation of PIK was required in NiCl₂-induced response. The finding that the treatment with PIK inhibitor wortmannin strongly decreased platelet

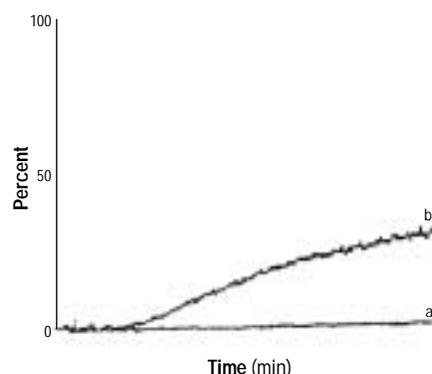


Figure 6. Platelet aggregation patterns of wortmannin-treated (10 µM for 15 min at 37°C) platelets in the presence of fibrinogen (1 mg/mL) in response to (a) 1 mM NiCl₂ and (b) 5 mM NiCl₂. The figure is representative of three experiments performed.



Figure 7. Translocation of the p85 subunit of PtdIns 3-kinase to the low-speed (15,600 × g) Triton X-100 insoluble cytoskeleton fraction of NiCl₂-stimulated platelets. Each reaction was stopped at the indicated time points. Platelet cytoskeleton proteins were separated by SDS-PAGE, and Western blots were performed using an anti-p85 polyclonal antibody. The figure is representative of three experiments performed.

aggregation in response to NiCl₂ indicated a role for PIK in NiCl₂-induced response.

PtdIns 3-kinase is known to translocate from the cytosol to the membrane cytoskeleton in the absence of fibrinogen only after vWf stimulation of platelets, with the ensuing activation of GpIb-V-IX complex, whereas stimulation with agonists such as ADP, epinephrine, or collagen has no such effect (14). In fact, vWf-induced cytoskeletal association of PIK and pp60^{src} occurs despite treatment with RGDS or EDTA, which disrupts the ligand-binding capacity of αIIbβ3 but does not affect the ability of vWf to bind GpIb-V-IX (21,22). Moreover, this last binding is specifically blocked by an anti-GpIb monoclonal antibody, and it is not observed in platelets lacking the glycoprotein Ib/IX complex (Bernard Soulier syndrome) (14). To verify whether NiCl₂-induced platelet activation proceeds through its binding to platelet GpIb-V-IX, we studied the cytoskeletal localization of PIK after NiCl₂ addition in the absence of exogenous fibrinogen. The results demonstrated that nickel was able to cause PIK translocation in a time-dependent manner.

The fact that RGDS had no inhibitory effect on NiCl₂-induced cytoskeletal association of PtdIns 3-kinase strongly supports the idea that NiCl₂ acts in a vWf-like manner. Our data confirm that this step, far from being dependent on αIIbβ3 binding to fibrinogen, involves other integrins, possibly GpIb.

Taken together, these observations suggest the existence of two phases in platelet response to NiCl₂ stimulation: a first step represented by the initial contact and binding to GpIb-V-IX complex, associated with cytoskeletal reorganization and translocation of PIK, and a second leading to the conversion of GpIIb-IIIa to an activated state necessary to support platelet aggregation. To the best of our knowledge, this is the first evidence of a direct role of NiCl₂ in inducing receptor activation in platelets.

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